

THE ABILITY OF NEW INTRACANAL MEDICAMENTS TO PREVENT THE
FORMATION OF MULTI-SPECIES BIOFILM
ON RADICULAR DENTIN

by

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INTRODUCTION

Regenerative endodontics is aimed at treating immature permanent teeth with necrotic pulps whose development has been curtailed by trauma or infection [1]. Regenerative endodontic procedures (REPs) incorporate everything necessary for successful tooth root formation: stem cells, scaffolds, and growth factors [2]. A review of regenerative endodontics by Murray found that modern advances in endodontics are bringing about a change in treatment of these teeth to achieve more predictable and successful results [3]. However, it has taken several years to develop predictable strategies for regenerative endodontics, and more research is needed.

Nygaard-Ostby was one of the first authors to discuss re-vascularization in necrotic endodontic cases. Nygaard-Ostby observed that the formation of a blood clot in the canal of a tooth with pulpal necrosis was important for adequate healing [4]. The author observed a resolution of pathologic symptoms in necrotic cases including, but not limited to, radiographic evidence of apical closure. This study also concluded that although desirable cells like fibroblasts were stimulated from the revascularization, or regenerative process, other cells that are not desirable were also present in the canal space. Other studies were conducted that included the use of intra-appointment disinfectants and inter-appointment medicaments without the formation of blood clot inclusion [5]. In 2001, Iwaya described a revascularization procedure on immature permanent teeth utilizing an antibiotic paste [6]. Current studies utilize the combination of antibiotics and the formation of intracanal clotting as described by Trope a few years

later [7]. These studies and several others laid the foundation for what endodontists today refer to as REPs.

Today the majority of endodontic research on immature teeth with necrotic pulps focuses on enhancing pulpal tissue growth and eliminating pathosis. Endodontic research is focused on this area to better achieve long-term success and survival in traumatized immature teeth. These immature teeth with necrotic pulps exhibit prematurely formed root structures with thin dentinal walls resulting in increased propensity of cervical root fracture and make for questionable abutments for prosthodontics due to poor crown/root ratios [8, 9]. Historical methods for treating these teeth included apexification.

Apexification is a method of utilizing intracanal calcium hydroxide ($\text{Ca}(\text{OH})_2$) to induce hard tissue formation as an apical barrier to confine the obturation material to the canal [10-12]. The obturation material of choice is mineral trioxide aggregate (MTA) because of its biocompatibility and sealing capabilities. Although this method of creating an apical barrier has had its success, it doesn't address the aforementioned problems associated with failure of continued apical and lateral root development, hence the need for regenerative endodontics.

Several treatment protocols are suggested and supported in literature for regenerative endodontics as stated by the American Association of Endodontists (AAE). The substance of regenerative endodontic treatment consists of disinfecting the canal space, followed by inducing bleeding into the canal by lacerating the apical papilla [7]. Traditionally, irrigating solutions and intra-canal medicaments are used during the regenerative endodontic procedure. The gold standard irrigating solution is sodium hypochlorite (NaOCl) due to its excellent antibacterial effect and ability to dissolve

organic tissue. However, its concentration is abbreviated to 1.5 percent to limit stem cell cytotoxicity [13]. As stated, Ca(OH)_2 has been used as an intra-canal medicament, but the current trend is focused on double and triple antibiotic pastes for canal disinfection.

Following disinfection, the establishment of bleeding into the canal space is accomplished via the use of a hand file, instrumented beyond the apex [14, 15], resulting in the eventual formation of a blood clot inside the canal that acts as a scaffold for the support of stem cells and growth factors like VEGF, EGF, and P1GF. This combination of disinfection, scaffolding, growth factors, and stem cells frequently supports continued root development [2].

Disinfection is perhaps the most important step in order to achieve a predictable outcome. Disinfection during an appointment is accomplished with 1.5-percent NaOCl as previously mentioned. Optimization between appointments can be accomplished via several medicaments. There are three major regenerative intra-canal inter-appointment medicaments: Ca(OH)_2 , triple antibiotic paste (TAP) and DAP. Ca(OH)_2 has shown effectiveness due to its alkalinity; which allows lysis of lipopolysaccharide [16]. DAP is of interest in recent endodontic literature as an intra-canal medicament and is one of the least studied. This thesis will focus on the use of DAP. TAP will be discussed first to get a better understanding of the importance of DAP.

TAP, composed of ciprofloxacin, metronidazole, and minocycline has been widely used as a canal medicament [7, 17, 18]. It is placed in the canal space for a period of one to four weeks [17, 19] because of its ability to produce predictable and desirable treatment outcomes [20]. Triple antibiotics are not without their weaknesses; these weaknesses include the tendency to discolor teeth, demineralize dentin, and create a

cytotoxic environment for stem cells [21-23]. Because of dentin discoloration due to minocycline in TAP, DAP has been utilized for disinfecting canals and promoting a favorable environment for regeneration of immature permanent teeth with necrotic pulpal tissue [24]. Although minocycline is omitted in the composition of DAP, cytotoxicity to stem cells can still occur at high concentrations [25]. For this reason, recommended concentrations of DAP have been reduced in current AAE guidelines [26]. One study focused on placing a clinical concentration of DAP in an immature tooth with necrotic pulpal tissue for several months; 30-month follow-up radiographs revealed apical closure and dentinal wall thickening [27]. Nevins and Cymerman supported this in a study in which they found a reduction in symptoms and in the size of periapical radiolucencies following the use of DAP. Additionally, Nevins and Cymerman concluded that periapical tissue repair was not adversely affected [28].

Comparison of *in-vitro* studies of DAP, TAP, and $\text{Ca}(\text{OH})_2$ against common oral pathogens such as *Enterococcus faecalis* and *Porphyromonas gingivalis* demonstrated that DAP was equivalent to TAP and superior to $\text{Ca}(\text{OH})_2$ in disinfection, while not discoloring the teeth as TAP has been shown to do [29]. In a study by Sabrah, the residual antibacterial effect of TAP exhibited significant antibacterial effects for up to 7 days using concentrations of 1 mg/ml and 0.5 mg/ml, while higher concentrations (1000 mg/ml) had a significant residual antibacterial effect for up to 14 days [24]. For DAP, significant antibacterial effects for up to 30 days were shown with concentrations as high as 1000 mg/ml, thus indicating a longer residual antibacterial effect of DAP compared with TAP. Sabrah concluded that the active antibiotics in DAP, ciprofloxacin and metronidazole, may bind dentin more effectively and/or absorb more readily into the

dentin surface [24]. A finding to note in the aforementioned study is that a concentration of 1 mg/ml of both TAP and DAP showed no significant difference in the residual antibacterial effect compared with 1000 mg/ml at all measured time points. However, the challenge of using a lower concentration is the difficulty in handling characteristics. Thus, delivery vehicles such as methyl cellulose may be useful in concentrations of 1 mg/ml or lower of DAP.

We are specifically interested in the residual antibacterial effect, or substantivity, of DAP. Previous studies on the substantivity of medicaments used in regenerative endodontics have focused on a particular bacterial species, commonly *E. faecalis* as studied by Sabrah et al. However, this single bacterium is unlikely to resemble the actual microbiota found in a clinical necrotic case. Endodontic infections are composed of multi-species in a biofilm [30]. Research by Nagata identified the most common endodontic bacterium to be *Actinomyces naeslundii* during regenerative treatment of immature teeth [31]. To further exemplify our need to focus on a multi-species biofilms, Tzanetakis et al. performed a study using pyrosequencing to determine the most abundant bacterial species in recurrent and primary endodontic infections [32]. The most to least abundant bacterial phyla were as follows: Bacteroidetes, Firmicutes, Actinobacteria, Synergistetes, Fusobacteria, Proteobacteria, Spirochaetes, and Tenericutes. The authors identified over 40 other species as well. Several authors have published studies investigating the various bacteria and the most likely species in endodontic infections, as shown above. The differences in bacterial biofilms and planktonic species found by various researchers are multifactorial. One suspected reason is dentinal tubule thickness. As we age, dentinal tubules calcify and mineralize, making bacterial penetration into the

tubules more difficult [33]. According to Kakoli and others, only about 60 percent of bacteria in endodontic species can penetrate into dentinal tubules [33]. Kakoli goes on to say that dentinal tubule thickness explains why eradication of bacteria from mature teeth is more predictable than in immature teeth. Kakoli explains that it is easier to eradicate a species when it cannot invade a space like the dentinal tubules; and that dentinal tubule thickness may provide insight as to why immature necrotic teeth can have a different bacterial population from a mature necrotic tooth. Thus, it is our desire to focus on multi-species in our study because research in this area is lacking. Our goal is to compare the residual effect of DAP against immature and mature teeth scheduled for endodontic therapy. To the best of our knowledge, no regenerative endodontic research utilizing DAP as the intra-canal medicament on dentin samples has focused on multi-species biofilms. Additionally, very few studies have looked into the residual antibacterial effect of medicaments used in regenerative endodontics. Methyl cellulose will be used as the delivery vehicle for the various concentrations of DAP. Methyl cellulose has been well documented in cases where TAP and DAP have been evaluated. This study may provide valuable information regarding the efficacy of DAP's substantivity on multi-species biofilm utilizing methyl cellulose as our vehicle

Objective:

- Specific Aims: To investigate the residual antibacterial effects of dentin treated with 1 mg/mL and 5 mg/mL of DAP against multi-species bacterial biofilms from immature and mature root canals.

Hypotheses:

- Null: All tested concentrations of DAP will not prevent the formation of multi-species biofilms regardless of the biofilm source.
- Alternative: All tested concentrations of DAP will significantly prevent the formation of multi-species biofilms regardless of the biofilm source.

REVIEW OF LITERATURE

HISTORY OF ENDODONTICS

The earliest dental texts can date back to approximately 5000 BC, when ancient Sumerians attributed toothaches to “tooth worms.” This theory was popular until it was finally refuted in 1684 by Anton Von Leeuwenhoek. Van Leeuwenhoek microscopically observed tooth samples and found microorganisms. A few years later in 1687, Charles Allen published the first English language book devoted to dentistry. This book was the first of its kind and outlined treatment options which included removing the rotten teeth and replacing them with sound ones [34].

Less than a century later scholars were looking for ways to achieve tooth-related pain relief while simultaneously maintaining the dentition. Treatments included mechanical and chemical pulpal remedies, as well as obturation of the pulp chamber. Around this time, the founder of modern dentistry, Pierre Fauchard, was the first person to present many of these ideas [34, 35]. Fauchard described many dental and endodontic basics in his book, *The Surgeon Dentist*. One important concept that Fauchard addressed was the pulp chamber access hole to allow for drainage. He also suggested using lead foil as an obturation material. This made Fauchard the first to discuss obturation in endodontics. In Germany less than three decades later, Philip Pfaff discussed pulp capping using gold or lead [36]. In 1756 Bourdet described endodontic therapy as a method of intentional extraction followed by replantation in an attempt to save the nerve [34].

It wasn't until the mid-18th century when Robert Woofendale became the first US resident to perform endodontic procedures. Woofendale cauterized the pulp to alleviate

pulpal pain. He also proposed the use of oil of cinnamon, cloves, turpentine, opium, and camphor to stymie pulpal pain [37, 38]. Later in the century, Frederick Hirsch described percussion testing as a diagnostic measure for periapical disease [35].

The first 50 years of the 19th century was devoted to the “vitalistic theory.” During this time, pulpal and periradicular physiology were of interest to dental practitioners. These dentists began to understand the importance of pulpal vitality for patient and dental health. This 50-year stretch also garnered attention for the introduction of pulpal anesthesia and new dental instruments [34]. In 1805 J.B. Gariot introduced the concept of pulp vitality as well as the ability to maintain non-vital teeth [39]. Less than 4 years later, in 1809 Edward Hudson became the first person to place gold foil into canal space [40]. A decade later Charles Bew introduced the concept of pulpal circulation. This concept focused on allowing blood to flow through the apical foramen into the pulpal space [39]. In 1826 Leonard Koecker wrote *Principles of Dental Surgery*. In his book, Koecker challenged the paradigm that a non-vital tooth could be maintained and further claimed that pulpal extirpation would lead to the death of the tooth like a foreign object. Thus, Koecker promoted prevention of pulpal necrosis via the use of pulp capping procedures similar to that outlined by Pfaff [38, 39, 41]. Only a few years later, SS Fitch described the “vitalistic theory” in his book, *System of Dental Surgery* in 1829. Fitch explained that the entire tooth is vital, citing long bones as an example. To paraphrase Fitch, the crown of the tooth was supplied by pulpal circulation alone, while the root was supplied by both pulpal circulation and periodontal ligament. As a result of Fitch’s discovery, pulpal extirpation and decoronation became popular. These teeth were restored with crowns, albeit poorly fitting. During the same time, a second and contrary school of

thought advocated “nonvitalistic theory.” These practitioners believed that enamel and dentin were devoid of any circulation, sensibility, and self-repair capabilities. These practitioners believed that pulpal extirpation would not affect tooth health [39]. With the two contradictory schools of thought, advancements came about over the next several decades, including the use of medicaments. Shearjashub Spooner started advocating arsenic trioxide to devitalize the pulp in combination with pulpal debridement prior to extraction. The use of arsenic trioxide is extremely inflammatory to host tissues, but was widely advocated because of its association to pain relief [35, 42, 43]. Jacob and Joseph Linderer used narcotic oil as a means to anesthetize the pulp in 1837[36]. The first endodontic broach was developed in 1838 by Edwin Maynard[44]. A year later, Baker wrote the first complete account of root canal therapy, which in essence summarized the first half of the 19th century.[35]

The latter half of the century saw changes with new instruments, disinfectants, obturation materials, surgical endodontics, and diagnostic testing. Obturation in 1850 was performed via the use of creosote-soaked wood plugs [42]. In the same year, Codman stated that the goal should be secondary dentin over the pulp [39]. And a year later, Hullihen described the first endodontic surgical procedure. Hullihen examined flap reflection, osteotomy, and tooth trephination to induce hemorrhage as a means of pain reduction [38]. Jonathan Taft and others believed that reparative dentin was more resistant to decay than natural dentin structure [36]. In 1864 rubber dam isolation was proposed by Sanford Barnum [42, 43]. In 1867 Dr. G. A. Bowman introduced gutta-percha as the obturation material [43]. At the same time, Clarke Dubuque introduced applying heat to gutta percha [34], and Magitot introduced applying an electric current

for pulpal vitality testing [45]. In 1870 G. V. Black, who advocated “extension for prevention,” recommended the use of zinc oxychloride as a pulp capping material [35]. It wasn’t until 1878, when Dr. G. O. Rodgers suggested pathogenic microorganisms as pulpal contaminants. A year later the vitality theory shifted to a septic theory. The septic theory explained how teeth become infected and treatment should be focused on teeth as the source of infection [39]. Believing in the septic theory led to one adopting asepsis/disinfection of the canal space in an effort to mitigate pathogenic microorganisms [46]. Canal space asepsis using antiseptic agents was purposed by Arther Underwood. [34]. Rounding out the 19th century, Bowman advocated chloropercha, a combination of gutta percha and chloroform as an obturation form. [42].

The 20th century saw more advances in endodontics than any other time in history. Some of these advances included anesthesia. The focal infection theory was popular for a time, and research provided a better understanding of bacteria and canal shape/size. Procaine (Novocaine) was developed in 1905 by Einhorn. Until that time, cocaine was the only other anesthetic agent. The use of nerve blocks during anesthetic delivery became widely used [43, 44]. Radiographs were introduced in 1913, and were commercially sold in 1919 [47]. Radiographs gave clinicians a two-headed approach to understanding periapical disease [48]. These radiographs allowed clinicians to establish a working length and apical size during instrumentation [46, 49]. Radiographs greatly enhanced our knowledge and understanding of root canal anatomy.

In the early part of the turn of the 20th century, the focal infection theory was popularized. This theory claimed that multiple diseases could stem from one foci of infection [50, 51]. The focal infection theory was proposed by William Hunter, a

pathologist and physician at McGill University in Montreal, who published a paper entitled “The Role of Sepsis and Antisepsis in Medicine,” which was published in *Lancet* in 1911 [47, 51]. William Hunter stated that gold fillings, caps, crowns, bridges, and crowns that are near a diseased tooth will form a gold mausoleum over a mass of sepsis. [50]. This theory led to the extraction of non-vital teeth as a means of preventing infection in other locations of the body [51, 52]. Fortunately, studies conducted in the 1930s and 1940s showed no conclusive relation between dental infection and systemic disease [37]. Tunicliff and Hammond demonstrated microorganisms can be found in pulps without disease [46, 49]. The role of these studies led researchers to disregard the focal infection theory, and this signified the beginning of the “scientific era.” [47].

Dr. Alexander Fleming garnered world-wide recognition for developing penicillin. Drs. Adams and Grossman discussed antibiotic use as an adjunct to root canal procedures in the 1940s [47]. Grossman talked about intracanal dressings with antibiotics. This sparked an interest in chemotherapeutic treatment of root canals; however, it was soon realized that antibiotics alone could not entirely render the canal space aseptic. Thus, the process of chemo-mechanical preparation was developed to create a canal space to allow chemicals to disinfect the canal space [53].

The American Association of Endodontists (AAE) was organized in Chicago during WWII. However, the field wasn’t thought of as a specialty until 1949, and as a result, the American Board of Endodontics (ABE) was formed in 1956 [54]. Through the hard work of its members and leaders, and as a result of the growing number of board certified specialists, the American Dental Association recognized endodontics as a specialty in 1963 [47].

Towards the end of the 1900s advancements were seen in the field of endodontics. Some of the greatest advancements were seen with nickel titanium files, microscopes and the illumination that coincides with better visualization, cone-beam computed tomography, and biomaterials like mineral trioxide aggregate (MTA) [55-60]. Also, the field of regenerative endodontics gained wider recognition. The first regenerative endodontics conference was held at Nova Southeastern University in 2006 [61]. In order to learn more about this topic, the AAE dedicated \$500,000 to 29 regenerative projects at 13 different institutions between 2001 and 2010 [62]. Pulpal regeneration code D3354 was added in 2012 under the ADA Current Dental Terminology [63]. The concept of regenerative endodontics has become one of the most widely studied topics in most recent publications, and yet there is still a lot of knowledge to be gained.

THEORY OF ENDODONTICS

All endodontic procedures can be summed up in the study by Kakehashi, Stanley and Fitzgerald in 1965. This study demonstrated that in bacteria-free rats, exposed pulps can retain vitality. Neither trauma from eating or exposure to the oral cavity caused pulpal necrosis in these germ-free rats [64]. In 1981 Moller et. al. used monkeys to demonstrate that periapical inflammation was the result of infected pulp tissue and not necrotic tissue along [65]. Thus, these studies and many others like it provided the foundation for explaining the pathogenesis of pulpal and periapical pathology in endodontics. The goal of endodontics is to decrease the microbial load to a level sufficient for the body to eliminate pathosis while simultaneously restoring tooth function [66-69]. Insufficient reduction in the microbial load in the pulpal canal(s) can lead to periapical pathosis [70].

Endodontic authors have outlined steps they believe have the greatest impact on success versus failure [66, 67, 71, 72]. Dr. Stewart demonstrated three phases of endodontic therapy, including chemomechanical preparation, asepsis, and obturation [71]. The chemomechanical process of shaping and irrigating to render the canal aseptic while offering a space for obturation is the most crucial step. Grossman identified several principles to follow when performing root canal therapy. These include: maintaining clinical asepsis; retaining instruments inside the canal space; never forcing instruments; enlarging canal space to accommodate obturation material; continuous irrigation throughout treatment; irrigation solutions should remain in the canal space at all times; no additional treatment is needed for fistulas; a negative culture should be confirmed prior to obturation; obturation should be air-tight to prevent leakage; obturation should not be inflammatory to tissues or membranes around the tooth; treatment for an abscess is primarily achieved via proper drainage; avoidance of injection into infectious spaces; there are cases where healing of necrotic teeth is best managed surgically.

Herb Schilder stated that the most important objective in treatment is elimination of bacteria and its associated contents. Schilder also introduced the concept of 3-D obturation of the entire canal system [66]. He went on to say that a proper seal is a hermetic seal, which does not allow tissue fluids to flow into or out of the canal space. The three phases of root canal therapy are discussed in more detail in the following paragraphs.

CHEMOMECHANICAL INSTRUMENTATION

Chronologically, this is step number one. Instrumentation is defined as the process of enlarging the canal space to allow for irrigation solutions and later obturation

material to occupy the space [72-74]. Negotiating the original shape of the canal to the apical extent is desired. Undesirable consequences would include deviation from the original canal space during enlargement [72, 75]. Although instrumentation can reduce microbial load, it has been shown that as much as 35 percent of the pulpal canals remain untouched [76-78]. Additionally, studies have shown bacterial penetration into the dentinal tubules as much as 300 μm [79, 80]. These facts taken together highlight the importance of irrigation to render the rest of the canal space aseptic.

CHEMICAL IRRIGATION

Phase two of the root canal process involves using disinfecting solutions continuously to rid the canal space of harmful bacteria [81]. The gold standard irrigant is NaOCl due to its ability to dissolve organic tissue, lubricate the canal, and disinfect the space [82]. The pH of NaOCl is above 11. This allows for hypochlorous acid to disrupt oxidative phosphorylation in cellular processes, block membrane activities, and DNA synthesis [83-85]. There are many factors that alter the efficacy of NaOCl including time, temperature, and concentration to name a few [81, 86, 87]. A potential drawback of NaOCl is in its inability to eliminate inorganic smear layer that forms from mechanical instrumentation [88, 89]. Studies have shown that a single minute of EDTA adequately removes the smear layer [90]. The alternating use of irrigants like NaOCl and EDTA allows for deeper penetration into dentinal tubules where bacteria can reside; this can also lead to an improved hermetic seal [91, 92]. NaOCl is unable to provide residual effects or disable endotoxins [93-95]. In such cases, disinfection with chlorhexidine gluconate (CHX) is recommended. Chlorhexidine provides substantial bacterial load reduction for several weeks, working via electrostatically binding to bacteria and disrupting cell wall

integrity [96, 97]. The combination of NaOCl and CHX forms a precipitate that can be potentially dangerous to the patient. The precipitate is Para-chloroaniline (PCA), and can be prevented by using another irrigant to flush the canal [98, 99].

OBTURATION

Providing a hermetic seal of the canal to prevent the space to be occupied by bacteria is called obturation. This final step should not be irritating to tissues or membranes [72]. Studies have shown that obturation should be confined to the canal system and terminate within 1 mm of the radiographic apex and be consistent without voids [100, 101]. Endodontic sealer is used in addition to the obturation material to aid in providing this hermetic seal [100].

MICROORGANISMS

Bacteria that occupy the canal space and lead to endodontic infections are classified as primary or secondary, depending on whether a root canal has been completed prior to infection. Bacteria in an endodontic infection tend to form biofilms [102]. Studies have found that primary endodontic infections consist most commonly of gram-negative anaerobic rods [66, 103]. According to Nagata, immature teeth on average have 2.13 species per root canal, and the most commonly isolated species is *A. naeslundii*, a facultative, anaerobic, gram-positive rod [31]. *A. naeslundii* has also been isolated in the oral cavity and its pathogenicity corresponds to its activation of the innate immune system to trigger an inflammatory process [104-109].

Another commonly encountered endodontic species is *F. nucleatum*. *F. nucleatum* is a gram-negative rod found in combined endo-perio lesions [109, 110]. The unique

ability of *F. nucleatum* is due to its inherent ability to aggregate other bacteria leading to further biofilm development. *F. nucleatum* leads to further periodontal destruction around root apices. It accomplishes this task by interfering with the host's immune system. This is similar to *P. gingivalis*, in that virulence factors alter the host's response [111-113]. *P. gingivalis* is a gram negative anaerobe. It historically was classified as a black-pigmented species. These species are unable to maintain vitality with NaOCl rinse [114]. According to Nair, there are an average of 1.3 species per root canal [115].

The most widely studied endodontic species is *E. faecalis*, a gram-positive facultative anaerobe that can be isolated from primary and more commonly persistent or secondary endodontic infections [116]. The virulence of *E. faecalis* rests in its ability to invade dentinal tubules and avoid the beneficial effects of Ca(OH)_2 [102, 111, 115, 117]. The virulence of *E. faecalis* makes it difficult to eradicate from the canal system and periapical tissues [118].

IMMATURE TEETH WITH PULPAL NECROSIS

Immature teeth in pulpal necrosis provide a challenge for endodontists. The challenge resides in the thin dentinal walls and open apices that compromise long-term prognosis and make obturation confined to the canal space difficult [8, 9, 119, 120]. Thus, because of the curtailed development of the root apices, these teeth present many challenges. Currently, much research delves into the knowledge gap that is regenerative endodontics, as will be discussed later.

OBTURATING TEETH WITH OPEN APICES

Apexification was introduced in the 1960s as a procedure to obturate non-vital

teeth with immature apices by forming a calcified barrier via the use of the medicament Ca(OH)_2 [11]. The purpose of the calcified barrier is to prevent obturation material from extruding beyond the apex. After isolating the tooth, locating the canal(s), establishing working length, and disinfecting the canal, long-term Ca(OH)_2 is placed to form an apical barrier. Once an adequate matrix is formed, the canal is obturated [121].

A drawback to this procedure is that it doesn't allow for root wall augmentation in either thickness or length. Patient recall/compliance can also be problematic. Even with these limitations, the greatest disadvantage of these teeth is the propensity for cervical root fracture [122-125]. Cvek has reported cervical root fractures to be as high as 77 percent [8]. Modern apexification shortens the duration of calcium from multiple months to approximately four weeks. After the disinfection protocol, a membrane like Collatape or Collaplug is placed to obturate against. The modern apexification procedure is beneficial because it lessens the total treatment duration, preventing temporary restoration leakage; perhaps a large reason why success rates are around 90 percent [126-128]. Although success has improved with apexification modernization, the lack of root wall length or thickness poses the same risk, increased cervical root fracture. These factors have led to the emergence of regenerative endodontics.

REGENERATIVE ENDODONTICS

Regenerative endodontics has gained popularity recently due to success in using this technique to enhance and augment biological function [129]. Regenerative endodontics is composed of three processes: stem cells, scaffolds, and growth factors

[129]. Regenerative endodontics is a type of tissue engineering. It is accomplished via canal disinfection, inducing apical bleeding, and bringing pluripotent stem cells into the canal space to spark tissue growth [14, 20, 130]. The goal of regenerative endodontics is disinfection, replacing previously non-vital architecture with vital structures and tissues, and inducing root length and thickness [131].

TERMINOLOGY

The term regenerative endodontics was not one that came about overnight. Previously dubbed “revascularization,” regenerative endodontics is a more encompassing term. The term is better suited because not only is vascularity restored, but so is the pulp-dentin complex of cells [132, 133]. One of the tertiary goals, and great benefits to regenerative endodontics is the renewal of innervation to the tooth. The AAE defines the term Regenerative Endodontic Procedures (REPs) [134].

HISTORY OF REGENERATIVE ENDODONTIC PROCEDURES (REPs)

Nygaard-Østby began some of the primary work in the field of regenerative endodontics. This was in the early 1960s. Nygaard-Østby hypothesized that the formation of a blood clot in the canal would lead to revascularization and healing. To test his theory, Nygaard-Østby examined at 17 patients with vital and non-vital pulps. These patients received root canal treatment with apical enlargement to promote bleeding into the canal system. The teeth were restored with a permanent restoration and followed for up to three and one-half years. Teeth were eventually extracted and sectioned for analysis. All teeth had resolution of pathosis. Some showed apical closure, while others revealed ingrowth of connective tissue[4]. Histologically this tissue was different from

normal pulpal tissues; it lacked odontoblasts [133]. Approximately five years later, antibiotic pastes were developed as canal medicaments [133]. Myers conducted a study on mature teeth in monkeys and noticed periapical inflammation/root resorption. The takeaway from this study was that mature teeth cannot heal as sufficiently as immature teeth [135]. A short time later, Nevins investigated immature teeth with necrotic pulps which he treated with biochemical debridement and placed collagen-calcium phosphate gel for three months as an inter-appointment medicament. The teeth were sectioned and histologically evaluated. Histologically, the teeth demonstrated revascularization of the canal space with various forms of connective tissue like cementum, bone, and reparative dentin [136].

MODERN DISCOVERIES IN REPs

Iwaya published a case report in 2001 using double antibiotic paste (DAP) composed of ciprofloxacin and metronidazole to treat an immature tooth with pulpal necrosis and periapical pathosis [6]. Minimal mechanical instrumentation was performed and disinfection was achieved via 5-percent NaOCl and 3-percent H₂O₂. The tooth was lightly dried and DAP was placed for as inter-appointment dressing. DAP was later removed and Ca(OH)₂ was placed against the apical tissue. The tooth was restored with resin. Continued root growth and development was seen radiographically 2.5 years later [6]. Another case report was published 3 years later detailing the use of triple antibiotic paste (TAP) composed of ciprofloxacin, minocycline, and metronidazole [7]. The authors theorized that if an aseptic environment is created in other immature teeth with pulpal necrosis that revascularization may be predictably achieved. The case report that ensued was conducted using the same disinfection protocol followed by 1 month of TAP. At the

second appointment, apical bleeding was achieved by puncturing the apical tissues. The canal was allowed to fill with a blood clot, and MTA was used to seal the canal. At the two-year follow-up, the authors observed asymptomatic patients, continued root development, and vital pulp testing. Because of the observations, this treatment protocol was followed for multiple years because it yielded great success [137-140].

In 2005 the requirements for successful regenerative endodontic procedures were outlined: stem cells, growth factors, and scaffolding [141]. Lovelace had already described the stem cells as being pluripotent mesenchymal stem cells [14]. Banchs and Trope discussed in detail how introducing blood into the canal was pivotal because the clot provides the scaffold while the blood brings with it growth factors and other cells [7]. Kahler published a case series of 16 REPs where he found periapical healing in 90.3 percent of cases, and apical closure in 19.4 percent of cases at 1.5 years [142]. Root development was found to increase between 14.7 percent to 14.9 percent in width, and 25.1 percent to 28.2 percent in length [143, 144].

Histologic examination has been evaluated by several authors; the type of tissue ranges drastically. It has been reported that hard tissue without any vascularity and vascular tissue with bony islands have been identified. Wang identified three types of tissue in canine treated immature teeth: intracanal cementum, bone, and connective tissue. Shimizu additionally identified odontoblastic-like cells and loose connective tissue. And, Martin identified mineralized tissue and fibrous connective tissue without odontoblast-like cells during REP [6, 7, 22, 27, 145, 146].

INDICATIONS AND SUCCESSES

Regenerative endodontic procedures are primarily aimed at treated immature teeth with necrotic pulps. A partial reason for success is due to the apical diameter being larger than 1 mm leading to greater inflow of blood and growth factors [147]. A similar study by Laureys found that an apical foramen as constricted as 0.32 mm allowed for revascularization in canine teeth [148]. The definition of success has been vague over the coming of age in regenerative endodontics. For this reason, the AAE has defined three criteria to assess the outcome of regenerative endodontic procedures: 1) Periapical and periradicular healing with resolution of symptoms; 2) Continued development of root formation, and 3) Return of pulpal sensibility. The outcomes of REPs have increased significantly because of the foundation for success: clinical asepsis through disinfection, stem cells and growth factors, and the presence of a membrane or scaffold.

CLINICAL ASEPSIS THROUGH DISINFECTION

The primary study for endodontics dates back to Kakehashi, Stanley, and Fitzgerald. They showed using germ-free rats that in the absence of bacteria, no pathosis was allowed to transpire [64]. This principle is rudimentary to all endodontic procedures, and regenerative endodontics is no different, although it can be more challenging. In a dog study on immature teeth with pulpal necrosis, vital tissue was present only when clinical disinfection took place [149]. Like many others, this study confirmed the need for clinical asepsis, even in cases with immature teeth.

TYPES OF IRRIGANTS

Sodium Hypochlorite (NaOCl)

Sodium hypochlorite was first introduced in during 1919. It has stood the test of time and still is regarded as the primary irrigant for disinfection [150]. NaOCl is

antibacterial; it dissolves organic tissue, and it provides excellent lubrication for instrumentation [105, 151, 152]. The concentration of NaOCl varies with its application. An *in-vitro* study demonstrated the most effective concentration of NaOCl was 5.25 percent at 40 minutes to eradicate *E. faecalis* [153]. Due to its potential for causing harm to the host, in cases with open apices, the recommended concentration of NaOCl is 1.5 percent [154]. As stated previously, NaOCl can damage vital host tissue due to its excellent dissolving capability. This can adversely affect stem cells and growth factors in the apical papilla; thus the concentration is reduced to 1.5 percent [134, 155, 156].

Calcium Hydroxide (Ca(OH)₂)

A year after the introduction of NaOCl came the introduction of calcium hydroxide. Ca(OH)₂ has been shown to inactivate lipopolysaccharide (LPS) [16, 102, 157]. This is particularly useful when dealing with harmful secondary endodontic gram negative pathogens. Ca(OH)₂ works via direct contact by inhibiting DNA replication. Ca(OH)₂ is very alkaline, with a pH in excess of 12 [158]. The use of Ca(OH)₂ has enjoyed a long tenure in endodontics, and it continues to garner support.

Calcium hydroxide is recommended by the AAE for treatment in REPs. Ca(OH)₂ has shown reliability in creating a conducive environment for stem cells of the apical papilla (SCAP) in concentrations of 1 mg/mL [25, 144, 159, 160]. Ca(OH)₂ can also affect hard tissue formation by interfering with the osteoprotegerin/RANKL ratio [161]. Andreasen reported a 28-day inter-appointment canal dressing with Ca(OH)₂ can reduce tooth fracture resistance, and this was confirmed with other studies [122, 162]. Additional studies have addressed the limitations of Ca(OH)₂ at targeting specific endodontic

bacteria like *E. faecalis* and *P. gingivalis* species [29]. For this reason, antibiotic pastes have been suggested and studied.

Triple Antibiotic Paste (TAP)

Due to the multispecies bacterial component of endodontic infections, antibiotic pastes have been suggested. TAP was first reported by Hoshino after *in-vitro* endodontic studies comparing individual antibiotics and the combination of three different antibiotics used to make up TAP. TAP consists of ciprofloxacin, metronidazole, and minocycline [18, 163]. Metronidazole and ciprofloxacin work via inhibiting DNA synthesis. Minocycline targets protein synthesis. *In-vitro* studies demonstrated that 0.3 mg/mL of triple antibiotic paste significantly reduces endodontic bacterial load [17]. However, TAP is not without its limitations.

Intracanal antibiotics have a long track record of dentin discoloration, demineralization, and cytotoxicity to stem cells. Tetracyclines like minocycline, have been advised against in developing teeth because of irreversible dentin discoloration [22, 23]. Minocycline causes demineralization by binding to calcium ions with its low pH (2.9) [21, 164]. Recently, the effect of antibiotics on stem cells has been investigated because of cytotoxicity. Concentrations above 1 mg/mL have shown irreversible damage to SCAP, and thus lower concentrations have been recommended for REPs [25, 165].

Ethylenediaminetetraacetic acid (EDTA)

Ethylenediaminetetraacetic acid (EDTA) is a viscous chelator. It removes the inorganic portion of the smear layer [89]. The smear layer is created when mechanical

instrumentation packs dentinal debris on the walls of the canal. This layer occludes the dentinal tubules. EDTA chelates metallic ions and has the potential to cause bacterial cell death with time [102]. EDTA is of benefit in REPs for several reasons as discussed below.

Seventeen-percent EDTA irrigation has shown to be beneficial in removal of the smear layer and opening dentinal tubules. EDTA's removal of the smear layer allows for release of growth factors from inside dentin [166-168]. EDTA has also shown the ability to increase surface area with irregularities, and this could possibly lead to better stem cell attachment [169]. Dental pulp stem cells have shown association to dentin pre-treated with EDTA [170]. Additionally, EDTA has been demonstrated an ability to counteract the some of the deleterious effects of NaOCl in the canal system, potentially leading to increased survival of stem cells of the apical papilla [156]. One of the potential negatives of EDTA is that if left too long, it can demineralize peritubular and intertubular dentin [90]. A study by Teixeira demonstrated that a 1-minute rinse of 17-percent EDTA in straight canals followed by NaOCl inactivation resulted in sufficient removal of the intracanal smear layer [171].

STEM CELLS

All steps up to this point were aimed at disinfecting the canal system. Disinfection reduces the bacterial load to a manageable number for the body to eliminate pathosis. In order for canal growth, however, stem cells must be incorporated. This is the tissue engineering step of REPs. Stem cells can be either multipotent or pluripotent; they can divide into identical cells or into any human cell, respectively. Dr. Lovelace demonstrated a vast supply of stem cells in the periapical area of immature teeth with

necrotic pulps [14]. These cells will then differentiate into various pulpal cells. Stem cells can be harvested from apical papilla (SCAPs), dental pulp stem cells (DPSC), dental follicle progenitor stem cells (DFPCs), periodontal ligament stem cells (PDLSCs), and stem cells from human exfoliated deciduous teeth (SHEDs) [130, 172-176]. Stem cells are concentrated in the cell-rich zone of the pulp, near the odontoblastic layer [121]. The five types of cells described have shown promise for use in regenerative endodontic procedures and are essential for pulp fibroblasts, extracellular matrix, and collagen regeneration [121, 174, 177]. SCAP are believed to be the main source of undifferentiated cells involved in the process of root development [172].

SCAFFOLD

Growth factors and blood needs a substance to adhere to—this is the scaffold. The function of a scaffold is to provide an extracellular matrix that allows for transport of nutrients, oxygen, and metabolic waste to the site [141]. Nevins was the first to use a scaffold in REPs. This was in 1976 using a collagen membrane [136]. Many authors have suggested the use of a blood clot as the scaffold [149]. Hutmacher identified six properties of an ideal scaffold REPs [136]:

1. Porous structure to allow for tissue attachment.
2. Resorbable membrane.
3. Cellular growth and proliferation.
4. Sufficient mechanical properties.
5. Biocompatible materials.
6. Material must have good handling characteristics.

Traditionally, blood clots have been used as the scaffolding material. However,

with modern advances in medicine, new scaffolds have been investigated [131]. Platelet rich plasma (PRP) and/or platelet rich fibrin (PRF) has been suggested as a scaffold [137, 178-180]. Other authors have also identified several growth factors that can be released by these scaffolds [166, 167, 170].

GROWTH FACTORS AND ENDOGENOUS STEROIDS

Long-term corticosteroid use in canal systems have resulted in pulp chamber reduction [181]. Dexamethasone has been shown to increase dental pulp cell differentiation into odontoblast-like cells [16, 182]. In summation, growth factors increase the prognosis of REPs by providing necessary mediators for tissue engineering.

RECOMMENDED GUIDELINES FOR REPs

In 2016, the AAE released recommendations for regenerative endodontic procedures [26] The recommendations are as follows:

- Case Selection
 - Tooth with necrotic pulp and an immature apex.
 - Pulp space not needed for post/core, final restoration.
 - Compliant patient/parent.
 - Patients not allergic to medicaments and antibiotics necessary to complete procedure (ASA 1 or 2).

- Informed Consent
 - Two (or more) appointments.
 - Use of antimicrobial(s).

- Possible adverse effects: staining of crown/root, lack of response to treatment, pain/infection.
 - Alternatives: MTA apexification, no treatment, extraction (when deemed non-salvageable).
 - Permission to enter information into AAE database (optional).
- First Appointment
 - Local anesthesia, dental dam isolation and access.
 - Copious, gentle irrigation with 20-ml NaOCl using an irrigation system that minimizes the possibility of extrusion of irrigants into the periapical space (e.g., needle with closed end and side-vents, or EndoVac™). Lower concentrations of NaOCl are advised [1.5-percent NaOCl (20mL/canal, 5 min) and then irrigated with saline or EDTA (20 mL/canal, 5 min), with irrigating needle positioned about 1 mm from root end, to minimize cytotoxicity to stem cells in the apical tissues.
 - Dry canals with paper points.
 - Place Ca(OH)₂ or low concentration of triple antibiotic paste. If the triple antibiotic paste is used: 1) Consider sealing pulp chamber with a dentin bonding agent [to minimize risk of staining] and 2) Mix 1:1:1 ciprofloxacin: metronidazole: minocycline to a final concentration of 0.1 mg/ml to 1.0 mg/ml. Triple antibiotic paste has been associated with tooth discoloration. Double antibiotic paste without minocycline paste or substitution of minocycline for other antibiotic (e.g., clindamycin;

amoxicillin; cefaclor) is another possible alternative as root canal disinfectant.

- Deliver into canal system via syringe
- If triple antibiotic is used, ensure that it remains below CEJ (minimize crown staining).
- Seal with 3-4mm of a temporary restorative material such as Cavit™, IRM™, glass-ionomer, or another temporary material. Dismiss patient for 1 week to 4 weeks.
- Second appointment (1-4 weeks after 1st visit)
 - Assess response to initial treatment. If there are signs/symptoms of persistent infection, consider additional treatment time with antimicrobial, or alternative antimicrobial.
 - Anesthesia with 3-percent mepivacaine without vasoconstrictor, dental dam isolation.
 - Copious, gentle irrigation with 20 ml of 17-percent EDTA.
 - Dry with paper points.
 - Create bleeding into canal system by over-instrumenting (endo file, endo explorer) (induce by rotating a pre-curved K-file at 2 mm past the apical foramen with the goal of having the entire canal filled with blood to the level of the cemento–enamel junction). An alternative to creating a blood clot is the use of platelet-rich plasma (PRP), platelet rich fibrin (PRF), or autologous fibrin matrix (AFM).

- Stop bleeding at a level that allows for 3 mm to 4 mm of restorative material.
- Place a resorbable matrix such as CollaPlug™, Collacote™, CollaTape™ over the blood clot if necessary and white MTA as capping material.
- A 3-mm to 4-mm layer of glass ionomer (e.g. Fuji IX™, GC America, Alsip, IL) is flowed gently over the capping material and light-cured for 40 s. MTA has been associated with discoloration. Alternatives to MTA (such as bioceramics or tricalcium silicate cements [e.g., Biodentine®, Septodont, Lancaster, PA]) should be considered in teeth where there is an esthetic concern.
- Anterior and Premolar teeth - Consider use of Collatape/Collaplug and restoring with 3 mm of a non-staining restorative material followed by bonding a filled composite to the beveled enamel margin.
- Molar teeth or teeth with PFM crown - Consider use of Collatape/Collaplug and restoring with 3 mm of MTA, followed by RMGI, composite, or alloy.
- Follow-up
 - Clinical and radiographic exam
 - No pain, soft tissue swelling or sinus tract (often observed between first and second appointments).
 - Resolution of apical radiolucency (often observed 6 mos. to 12 mos. after treatment)

- Increased width of root walls (generally observed before apparent increase in root length and often occurs 12 mos. to 24 mos. after treatment).
- Increased root length.
- Positive pulp vitality test response
- The degree of success of REP is largely measured by the extent to which it is possible to attain primary, secondary, and tertiary goals:
 - Primary goal: The elimination of symptoms and the evidence of bony healing.
 - Secondary goal: Increased root wall thickness and/or increased root length (desirable, but perhaps not essential).
 - Tertiary goal: Positive response to vitality testing (which if achieved, could indicate a more organized vital pulp tissue).

MATERIALS AND METHODS

HUMAN DENTIN SPECIMEN PREPARATION

After IRB approval was granted, adult human intact permanent premolars were collected and stored in 0.1-percent thymol solution at 4°C. The crowns of these teeth were cut at the cervical neck using a water-cooled diamond blade saw (Figure 2). Then the teeth were sectioned longitudinally in a buccolingual direction into two halves (Figure 3, Figure 4). Each flattened, or cut (pulpal) side root half was used as a dentin slab after standardization. These dentin slabs were secured in an acrylic plate with wax (Figure 5). The cut pulpal side of the sectioned dentin was polished sequentially with different abrasive papers in the order of 500 grit, 1200 grit, and 2400 grit using a StruersRotopol 31/Rotoforce 4 polishing unit (Struers, Cleveland, OH (Figure 6)). Dentin samples were rinsed with 1.5-percent NaOCl and 17-percent EDTA for 4 minutes to remove the smear layer as previously described in the literature [183] (Figure 9). All specimens were kept in water throughout the procedure to avoid dehydration. Dentin specimens were stylized and kept wrapped until use (Figure 7, Figure 8).

ANTIBIOTIC PASTE PREPARATION

Previous studies are well documented as to the diluted paste-like consistency of DAP at various concentrations, and the concentration of DAP in this study was prepared likewise [184, 185]. In summary, United States Pharmacopeia grade antibiotic powders compounded of equal portions of metronidazole and ciprofloxacin (50 mg and 250 mg) were dissolved in 50 mL of sterile water to yield DAP in concentrations of 1 mg/ml and 5 mg/ml, respectively. Next, 4 g of methyl cellulose powder (Methocel 60 HG, Sigma-

Aldrich, St. Louis, MO) was added to each DAP/methyl cellulose concentration and mixed for 2 hours using a sterile magnetic stir bar in order to create a homogenous paste (Figure 10). Methyl cellulose was added because pastes with low concentrations are unable to remain on dentin structures without washing out. The pastes had concentrations of 1 mg/dl and 5 mg/ml of DAP. Commercial grade Ca(OH)₂ (UltraCal XS, Ultradent, South Jordan, UT) and an antibiotic-free paste made of sterile water and methyl cellulose was prepared using the same method in order to obtain a control (aqueous methylcellulose).

EXPERIMENTAL GROUPS

A total of 120 dentin specimens were randomly assigned into the six experimental groups (n = 20 per group). Specimens were placed in 96-well plates for treatment (Figure 11). Each experimental group included 10 dentin samples inoculated with the clinically isolated mature necrotic adult biofilm, and the remaining 10 samples were inoculated with the clinically isolated immature regenerative bacterial biofilm.

HUMAN DENTIN SPECIMEN TREATMENT:

Specimens were sterilized in ethylene oxide. There were 20 dentin specimens for each of the six groups: Group 1, 1 mg/mL of DAP; Group 2, 5 mg/mL of DAP; Group 3, Ca(OH)₂; Group 4, Sterile water and methyl cellulose used as a placebo paste; Group 5, No treatment with bacteria; Group 6, No treatment/no bacteria. These dentin samples were treated with 200 µL of the aforementioned groups. The concentrations of DAP were allowed to treat dentin samples for 1 week at 37°C and 100-percent humidity to prevent dehydration. After the one-week period, the specimens were irrigated for one minute

with 5 ml of sterile saline followed by irrigation with 10 ml of 17-percent EDTA for 5 minutes. Samples were kept independently in phosphate buffered saline (PBS) for 1 week (Figure 12).

BACTERIAL COLLECTION

The two bacterial sample collections were performed during normal root canal treatment. One clinically isolated biofilm came during root canal procedure of an immature tooth with a necrotic pulp that was scheduled for endodontic regeneration treatment (IRB # 1510640949). The second clinically isolated biofilm came from an adult tooth with pulpal necrosis scheduled for conventional root canal therapy (IRB # 1510640949). The microorganisms isolated from these teeth served as the two clinically isolated biofilms. The biofilm collection procedure was the same for each sample. The teeth were isolated with a rubber dam. Both tooth surface and rubber dam were cleansed with 3-percent hydrogen peroxide solution and disinfected with 6-percent NaOCl solution. The coronal root canal access was achieved with the use of sterile round burs. The pulp chamber was disinfected using a swab soaked in 6-percent NaOCl solution. This solution was then inactivated with sterile 5-percent sodium thiosulfate. Samples were collected from the infected root canal by means of a #15K file with the handle cut off. The file was introduced 1 mm short of the apical foramen and an up-and-down filing motion was used for 30 seconds. Three sterile paper points were inserted separately into the root canal at the same working length and were left intra-canal for 1 minute in order to wick the tissue fluid. Both the file and paper points were placed into 2 mL of BHI-YE, vortexed to elute bacteria, grown anaerobically at 37°C for 48 hours, and frozen at -80 °C with 10-percent sterile glycerol until used.

BACTERIAL STRAINS AND MEDIA

Anaerobic blood agar plates (CDC, BioMerieux, Durham, NC) were used to initially grow and maintain the separate clinically isolated biofilms. An infusion broth of brain heart broth supplemented with 5 g/L yeast extract (BHI-YE) supplemented with 5-percent (v/v) of hemin and vitamin K was used to grow each bacterial biofilm at 37°C in an anaerobic environment using gas generating sachets (GasPak EZ, Becton, Dickinson and Co., Franklin Lakes, NJ) to produce the required environment.

BACTERIAL GROWTH ON ROOT SPECIMENS

Root specimens were removed from PBS and placed individually inside a well of a sterile 96-well plate with the pulpal surface facing outward (Figure 12). Then, 190 µl of fresh BHI-GE growth media and 10 µl of a two- day old culture of clinically isolated bacterial species from an adult mature necrotic tooth were added to 10 of the wells in each experimental group and incubated anaerobically at 37°C for 3 weeks before performing the antibacterial testing (Figure 14). The remaining 10 wells of each experimental group were inoculated with 190 µl of fresh BHI-GE growth media and 10 µl of a 48-hour culture of the clinically isolated bacterial sample from an immature tooth with pulpal necrosis. These wells were incubated anaerobically at 37°C for 3 weeks before enumerating the number of bacteria in the attached biofilm (Figure 13). Media were replaced every week during incubation.

CONFIRMATION OF THE POLYMICROBIAL NATURE OF THE BIOFILMS

Bacterial samples were collected from each bacterial source before and after three weeks incubation of the bacterial biofilm on dentin samples to confirm the presence of

both gram positive and gram negative bacteria within the biofilm (additionally, this was confirmed by our pilot study). Furthermore, one randomly selected infected dentin sample from each type of biofilm (untreated groups) was selected and processed for SEM visualization to confirm the polymicrobial nature of the biofilm. Additionally, the aliquot of the bacterial samples obtained at the time of collection were kept at -80 °C for PCR analyses in future studies.

BIOFILM DISRUPTION ASSAYS

After bacterial biofilms were allowed to grow for 3 weeks, each dentin sample was individually transferred into a fresh 1 ml tube of sterile saline. Tubes were sonicated for 20 seconds and vortexed for 30 seconds to detach biofilm cells (Figure 15). Biofilms that have been removed were diluted (1:10 and 1:1,000) and spirally plated on blood agar plates (CDC, BioMerieux (Figure 16, Figure 19)). Bacterial plates were incubated at 37°C for 48 h in 5-percent CO₂. The number of CFUs/mL was determined by using an automated colony counter (Synbiosis, Inc., Frederick, MD) using an average of 2 counts per agar plate (Figure 20).

STATISTICAL ANALYSIS

The effects of treatment and type of biofilm on bacteria counts were analyzed using two-way ANOVA. Pair-wise comparisons among the treatment combinations were made using the Sidak method to control the overall significance level at 5 percent for each set of comparisons.

SAMPLE SIZE

Based on previous data the coefficient of variation was estimated to be 0.5. With a sample size of 10 samples per treatment-biofilm combination, this study will have 80-percent power to detect a 2.7-fold difference between any two treatments within each type of biofilm, assuming two-sided tests conducted at an overall 5-percent significance level. If the interaction between treatment and biofilm is not significant, the study will have 80-percent power to detect a 1.9-fold difference between any two treatments.

RESULTS

MATURE VS IMMATURE BACTERIAL COUNTS

When comparing bacterial counts for mature teeth versus immature teeth, mature teeth had significantly lower counts for DAP 1 mg/ml and DAP 5 mg/ml (≤ 0.005). When comparing mature and immature teeth, there were no significant differences found in bacterial counts for control ($p = 0.99$), placebo ($p = 0.27$), or Ca(OH)_2 ($p = 0.66$). No bacterial growth was evident clinically or with the automated colony counter for the negative control. Table I summarizes these findings.

Comparing the Control

The control had significantly greater bacterial counts than DAP 1 mg/ml and DAP 5 mg/ml for immature teeth ($p < 0.05$). The control also had significantly greater bacterial counts than DAP 1 mg/ml and DAP 5 mg/ml for mature teeth ($p < 0.001$). The control was not significantly different from placebo or Ca(OH)_2 for immature or mature teeth ($p > 0.80$) in regard to bacterial counts.

Comparing the Aqueous Methyl Cellulose Placebo

In comparing bacterial counts of the aqueous methyl-cellulose placebo to DAP 1 mg/ml and DAP 5 mg/ml for mature teeth ($p < 0.001$), the placebo was found to have significantly greater bacterial growth. Additionally, the placebo had significantly greater bacteria than DAP 5 mg/ml for immature teeth ($p < 0.001$). When comparing the placebo to Ca(OH)_2 , the placebo was not different from Ca(OH)_2 for immature or mature teeth. Additionally, the placebo was different from DAP 1 mg/ml for immature teeth ($p > 0.90$).

Comparing Double Antibiotic Paste Groups

When looking at the bacterial counts among DAP groups, bacterial counts were significantly greater for DAP 1 mg/ml than DAP 5 mg/ml for mature and immature teeth ($p < 0.001$). The bacterial counts were significantly less for DAP 1 mg/ml than Ca(OH)_2 for mature teeth ($p < 0.001$) but not for immature teeth ($p = 0.96$). Additionally, bacterial counts were significantly less for DAP 5 mg/ml than Ca(OH)_2 for mature and immature teeth ($p < 0.001$). Figure 2 summarize the results of the different treatment groups to each other and the controls.

FIGURES AND TABLES

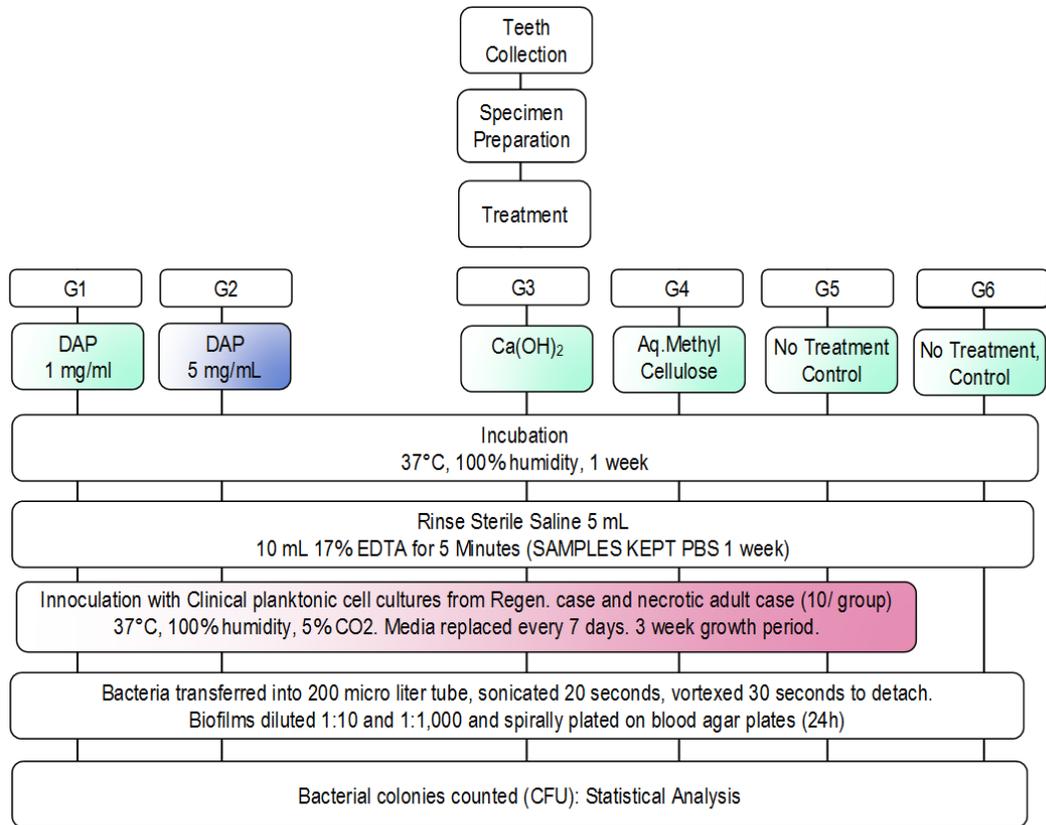


FIGURE 1. Experimental design flowchart. Note the two groups on the left are Double Antibiotic Paste with methylcellulose.

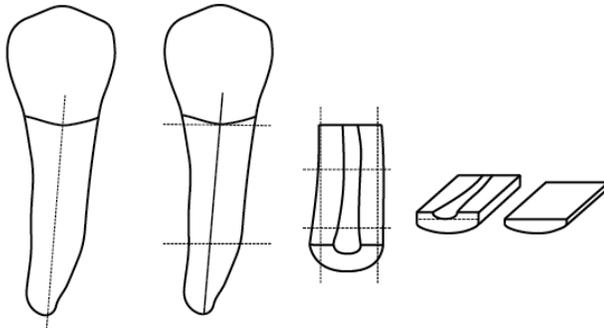


FIGURE 2. Picture depiction of specimen preparation. Each dentin specimen was sectioned to a 4x4x1mm standardized flattened pulpal surface.

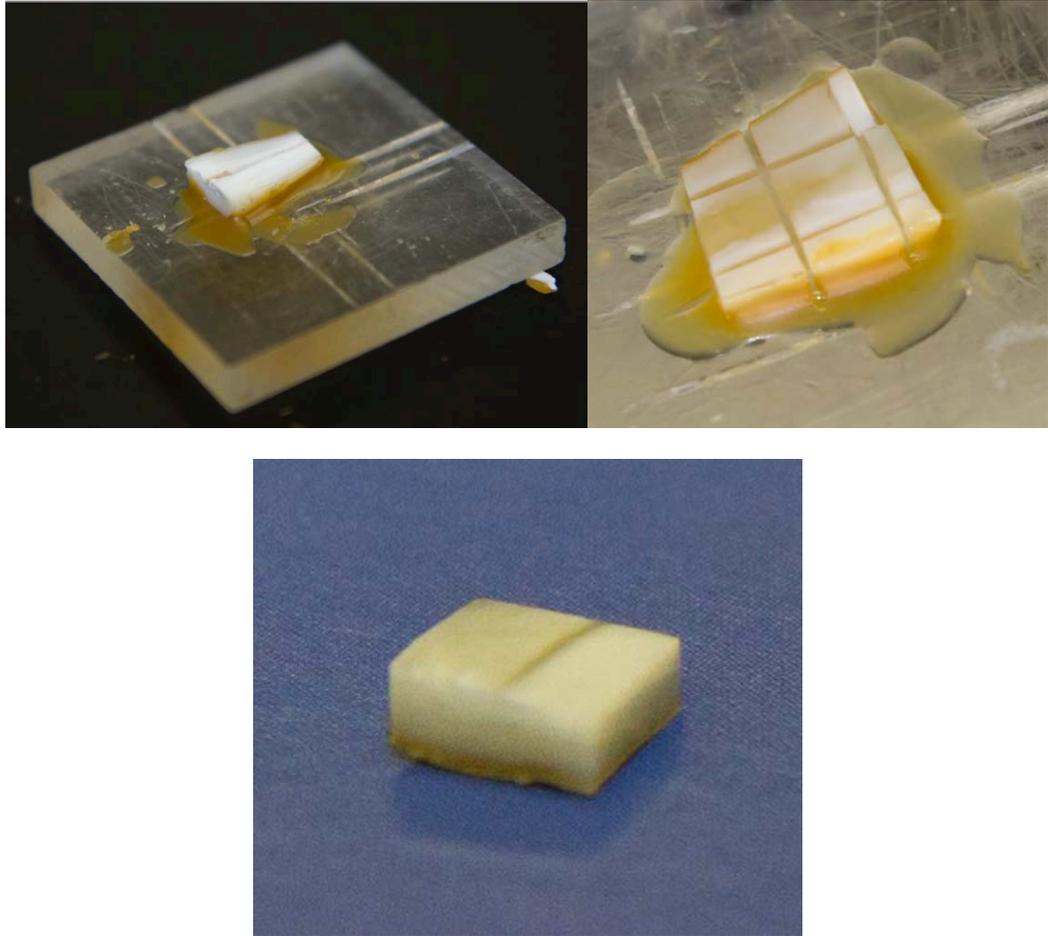


FIGURE 3. This is a clinical photo of Figure 2. Each half-root was cut into a 4x4x1-mm dentin sample with a double-bladed low-speed saw using water cooling. Note the pulpal side is up in the photos.

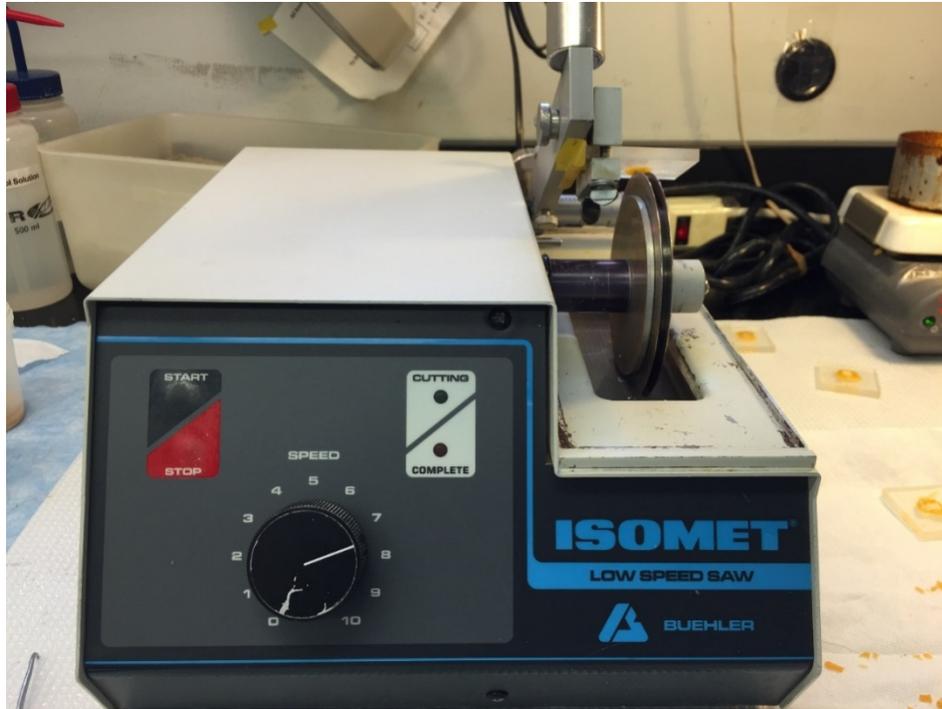


FIGURE 4. The low-speed double bladed saw used with water cooling. Spacers are used and dial calipers confirm 4x4x1mm standardization of dentin specimens.



FIGURE 5. Dentin specimens placed on cylinders were secured with wax prior to smoothing and polishing. This allowed for better uniformity and processing.



FIGURE 6. This RotoForce-4 unit was used to flatten, smooth and polish the pulpal surface of the dentin specimens to the 1mm height. Wetted abrasive papers were used in sequence to properly polish the pulpal surfaces.

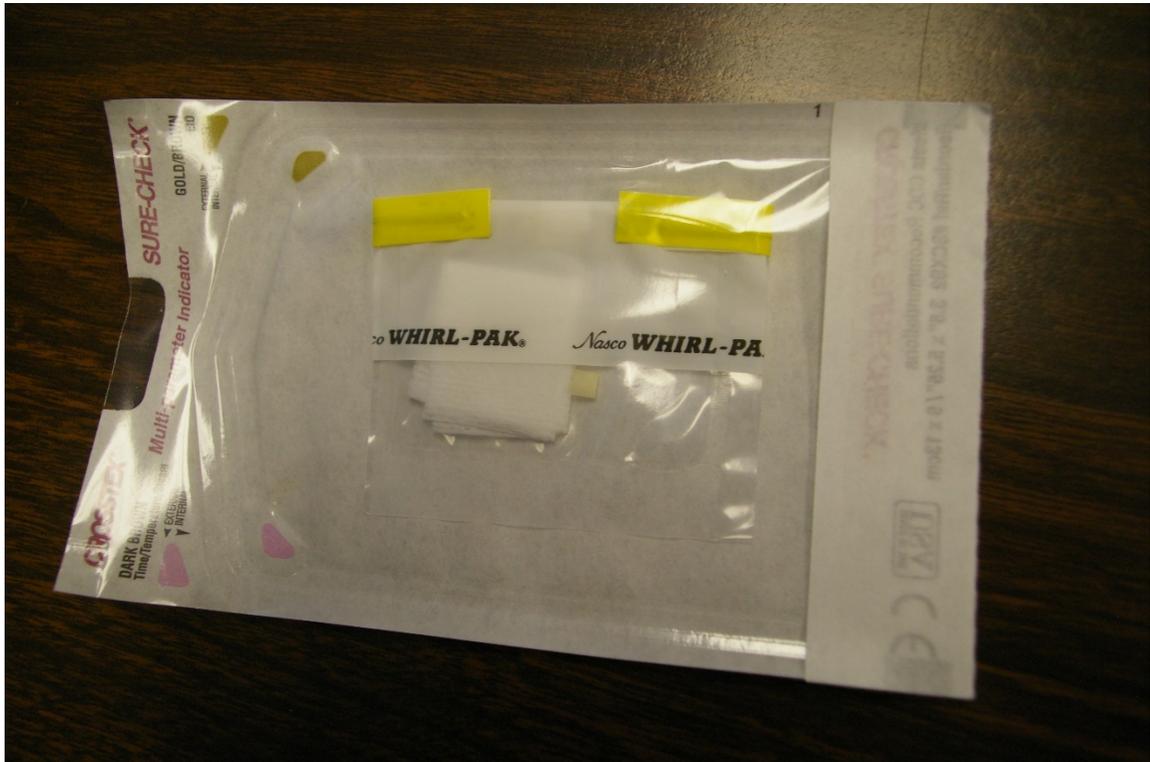


FIGURE 7. Sterile dentin specimens were individually wrapped to preserve sterility. These specimens were sealed with saturated sterile gauze to prevent dehydration. Dentin specimens were placed in a Whirl-pak, and used within a matter of weeks to ensure the best results.



FIGURE 8. Dentin specimen removed from individually wrapped packaging, showing polished 4x4x1mm surface.

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FIGURE 9. This is a ventilated hood where dentin unpackaging and treatment placement occurred. Long surgical pick-ups were used to keep pulpal surface facing upwards.



FIGURE 10. Prepared double antibiotic paste with methylcellulose used to get a homogenous paste-like consistency.



FIGURE 11. Dentin specimens were placed in sterile 96-well plates in prior to medicament placement. Figure above is prior to treatment. Note the clear wells.



FIGURE 12. Dentin specimens were placed in sterile 96-well plates for 1 week, and incubated prior to PBS immersion. Note the discoloration of the wells due to the BHI-YE.



FIGURE 13. All dentin specimens were treated for 1 week at 37°C with 100% humidity in the above incubator.



FIGURE 14. A sterile broth of brain heart infusion (BHI) supplemented with 5 g of yeast extract/L (BHI-YE) was used to supply the clinical species with growth media. Note the color. On the right is a sample of 48 hour clinical species; turbidity is evident—indicating bacterial growth.



FIGURE 15. Dentin samples were sonicated, vortexed, and sonicated to detach biofilm cells.



FIGURE 16. Spiral plating after dilutions of detached biofilms.



FIGURE 17. Incubation of spirally plated blood agar plates for 24 hours.



FIGURE 18. Blood agar plates incubated for 24 hours incubation after spiral plating. Note the bacterial colony formation on the agar plate.

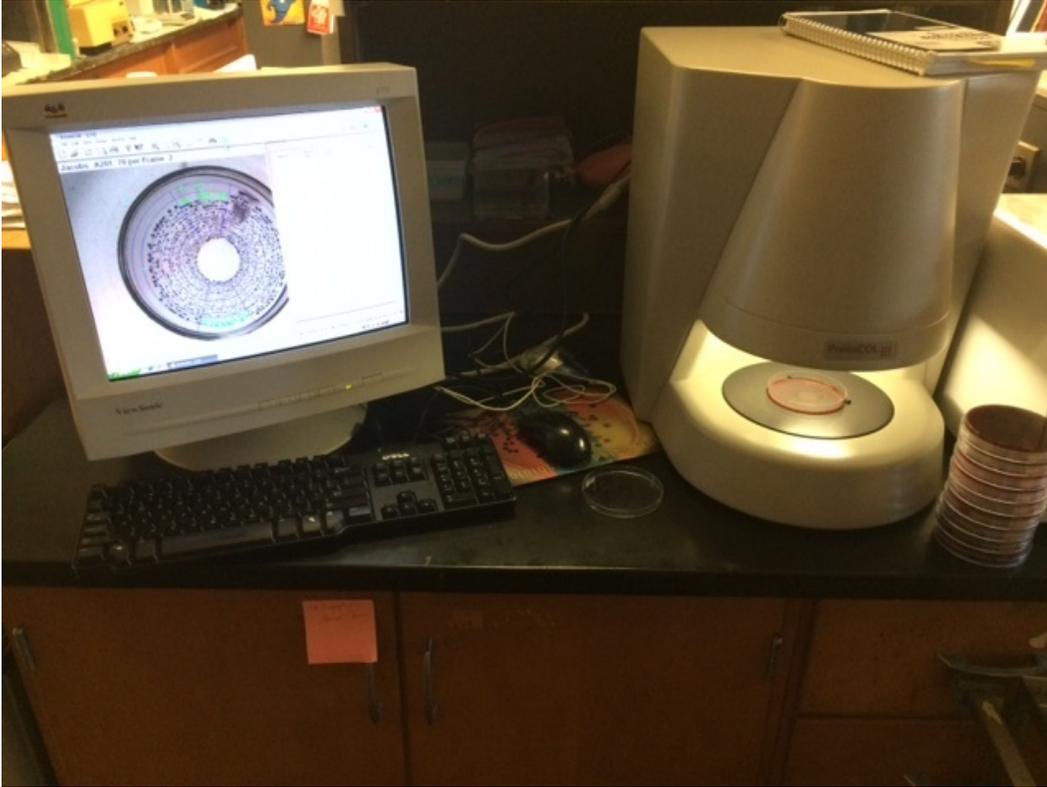


FIGURE 19. Blood agar plates were placed in automated colony counter after 24 hour incubation period to enumerate CFU/mL.

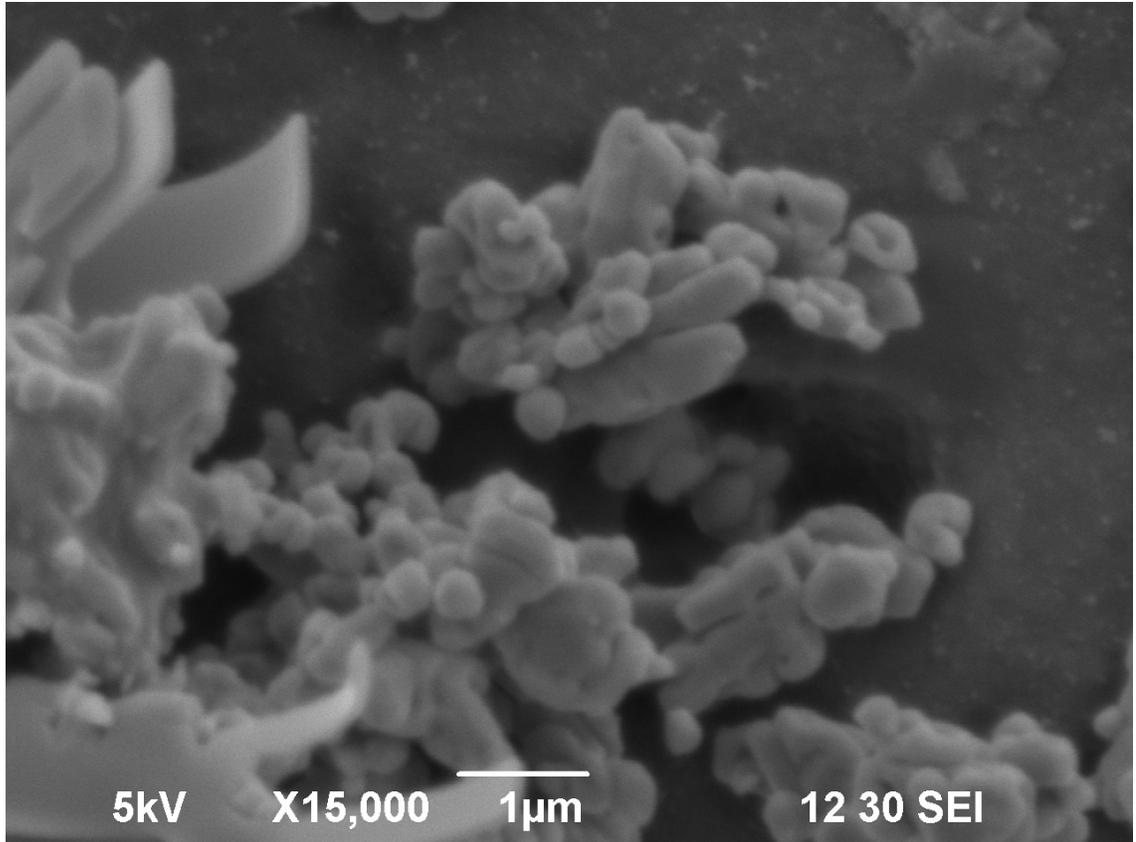


FIGURE 20. Scanning electron microscopic image of 3-week old bacterial biofilms formed on dentin surface. Biofilm formed by bacteria obtained from infected root canal of mature tooth with diagnosis of pulpal necrosis. Note the different shapes of bacteria in the SEM.

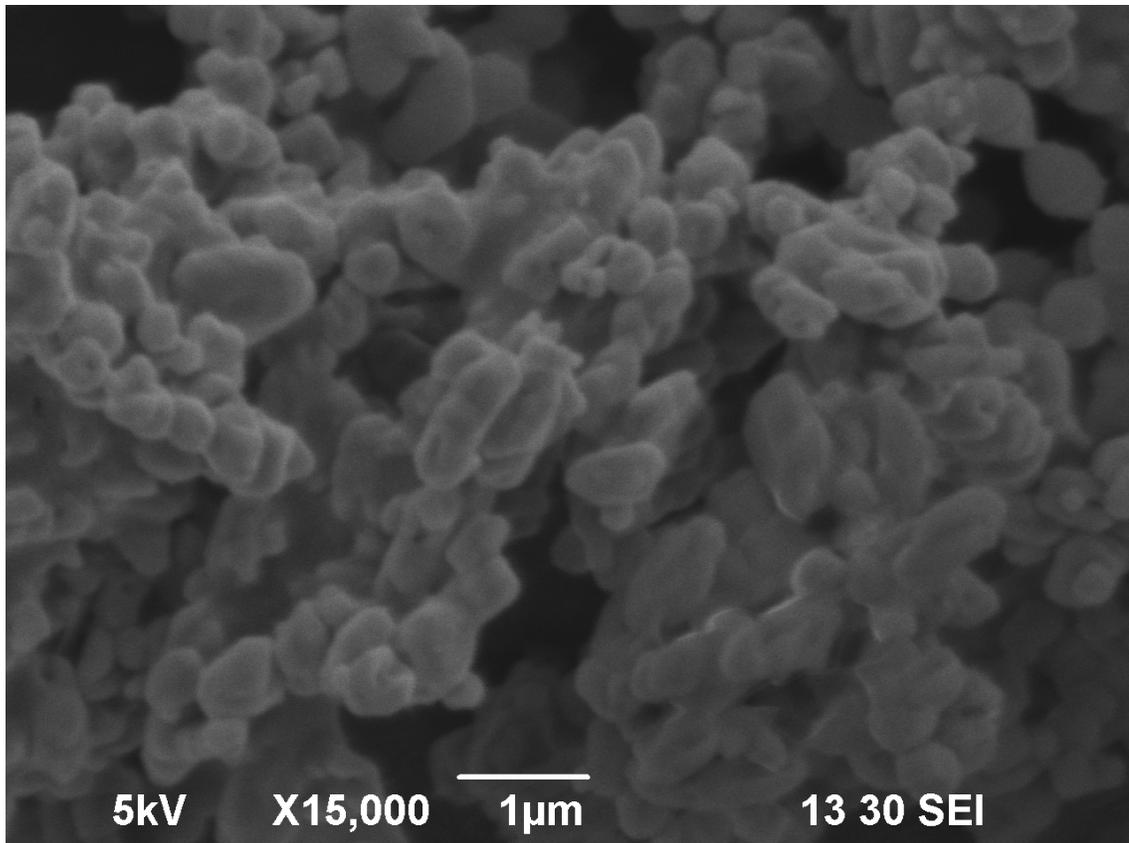


FIGURE 21. Scanning electron microscopic image of 3-week old bacterial biofilms formed on dentin surface. Biofilm formed by bacteria obtained from infected root canal of mature tooth with diagnosis of pulpal necrosis. Note the different shapes of bacteria in the SEM.

TABLE I

Tabulated results of mature vs. immature biofilm growth.*

Biofilm	Treatment	Mean (SE)
Immature teeth	Control	6.32 (0.10)
	Placebo	5.87 (0.08)
	DAP 1 mg	5.43 (0.15)
	DAP 5 mg	3.25 (0.21)
	CaOH	5.83 (0.13)
Mature teeth	Control	6.41 (0.13)
	Placebo	6.39 (0.07)
	DAP 1 mg	4.50 (0.10)
	DAP 5 mg	1.85 (0.47)
	CaOH	6.19 (0.13)

*Note: Tables present the Mean (SE) from the \log_{10} transformed data.

TABLE II

Tabulated comparison of biofilm growth.

Comparison type	Comparison	Subset	p-value
Biofilm	immature vs mature	control	0.999
		placebo	0.271
		DAP 1 mg	0.005
		DAP 5 mg	<.001
		CaOH	0.657
Treatment	control vs placebo	immature	0.898
		mature	1.000
	control vs DAP 1 mg	immature	0.034
		mature	<.001
	control vs DAP 5 mg	immature	<.001
		mature	<.001
	control vs CaOH	immature	0.816
		mature	1.000
	placebo vs DAP 1 mg	immature	0.903
		mature	<.001
	placebo vs DAP 5 mg	immature	<.001
		mature	<.001
	placebo vs CaOH	immature	1.000
mature		1.000	
DAP 1mg vs DAP 5mg	immature	<.001	
	mature	<.001	
DAP 1 mg vs CaOH	immature	0.956	
	mature	<.001	
DAP 5 mg vs CaOH	immature	<.001	
	mature	<.001	

DISCUSSION

Over the last two decades, regenerative endodontic studies have grown exponentially. The main components of regenerative endodontics are disinfection, scaffolds, growth factors, stem cells, and mediators. Our current research project is focused on the process of disinfection. This is the primary and key step in eliminating pathosis. However, care must be taken in order to avoid harm to the crucial structures and factors needed for growth and development.

Several *in-vitro* studies have focused on the proper concentration of medicament to eliminate pathosis while maintaining vitality to the stem cells and growth factors in the apical papilla [186-188]. Although some of these studies have shown promise in eliminating pathosis, the handling characteristics of low-antibiotic concentrated medicaments are a major deterrent to practitioners. In order to improve handling characteristics, as well as provide a stable and evenly concentrated low-concentration medicament, methylcellulose hydrogel was utilized in this study. Methylcellulose improves handling characteristics by raising the viscosity, while still allowing for an injectable intracanal dressing. Methylcellulose is currently utilized with $\text{Ca}(\text{OH})_2$ and has a proven track record due to its biocompatible nature [189, 190]. Methylcellulose has shown promise in previous residual antibacterial studies with DAP used as an intracanal medicament [191].

Guidelines set forth by the American Association of Endodontists (AAE) recommend the use of $\text{Ca}(\text{OH})_2$, or 0.1mg/ml - 1 mg/mL of TAP or DAP as the intracanal medicament during REPs [26]. The guidelines were derived from several

studies that focused on eliminating pathosis while causing minimal cytotoxic effects to stem cells and growth factors in the apical papillae [192-194]. This study focused on the residual effects of low concentrations of DAP as compared with previous studies, which focused on higher residual concentrations [191]. The reason for this current study was to assess the ability to eliminate pathosis, and maintain a level of clinical asepsis needed for regeneration because post-treatment contamination can significantly cause adverse effects. This thesis used bacteria clinically isolated from mature and immature teeth, which is different and more clinically relevant than previous studies which used standardized *E. faecalis* biofilms [187, 191].

The AAE recommends either Ca(OH)_2 , or 0.1mg/ml - 1 mg/mL of TAP or DAP as the intracanal medicament of choice [26]. DAP has been suggested over TAP because of the minocycline staining associated with TAP used as an intracanal medicament [23]. One advantage of DAP over Ca(OH)_2 , is the residual antibacterial effect of DAP; Ca(OH)_2 , only works when it is in direct contact [191]. This study has also demonstrated the residual capability of DAP in low concentrations. Some of the substantivity can be due to the challenge of DAP's removal from the dentinal walls as stated by Arslan [195]. Another study demonstrated a reduction in the push out bond strength between the root canal and various cements when DAP was applied, thus indicating DAP having substantivity [196, 197].

A previous study by Jenks demonstrated that only 50 mg/mL and 500 mg/mL of DAP treatment for one week were able to provide a significant and substantial reduction in bacterial counts for substantivity [191]. This study demonstrated that 1 mg/mL and 5 mg/mL of DAP exhibited significant and substantial residual antibacterial effects (\approx 2-4

\log_{10} reduction in CFU/mL) against bacterial biofilm obtained from mature teeth with necrotic pulp. However, only 5 mg/mL demonstrated significant and substantial residual antibacterial effects ($\approx 3 \log_{10}$ reductions in CFU/mL) against bacterial biofilms obtained from immature tooth with necrotic pulp. It should be stated that low concentrations of antibiotic pastes are advised over high concentrations previously recommended because of the reduced harm to apical stem cells [25, 193]. Additionally, this study demonstrated that dentin pretreated with 1 mg/mL and 5 mg/mL of DAP exhibited significantly greater residual antibacterial effects against bacterial biofilms obtained from mature teeth with pulpal necrosis than that obtained from immature teeth with pulpal necrosis. Thus, it can be deduced that bacterial biofilms from immature teeth with pulpal necrosis are more resistant to disinfection than mature teeth with pulpal necrosis. This may be related to dentin tubule diameter, or the specific bacteria isolated in these clinical cases. This is supported by a clinical study that looked at comparing mature and immature teeth with pulpal necrosis; the study found that immature teeth were more resistant to disinfection than mature teeth, regardless of the NaOCl concentration used [198]. It is reasonable to assume that more resistant endodontic pathogens are found in infected root canals of immature teeth with pulpal necrosis. A study by Nagata found that the most commonly encountered bacteria in immature teeth with pulpal necrosis are *A. naeslundii* and *Porphyromonas endodontalis* [199]. This is different from the most commonly isolated bacterial species found in mature teeth with pulpal necrosis, which is *Fusobacterium* and *Prevotella* species [200]. Again, these species are different from the commonly used *E. faecalis* biofilm. Thus, it can be deduced that our study is applicable because it used cultured bacteria from clinical cases.

In this study, comparing Ca(OH)_2 with double antibiotic paste demonstrated that Ca(OH)_2 was ineffective as a residual canal disinfectant against established clinical biofilms. This is supported by another clinical study [191]. Several studies confirm that the antibacterial ability of Ca(OH)_2 is pH dependent [201]. This is because extreme alkaline environments ($\text{pH} > 11$) provide inhospitable conditions for bacterial proliferation. The AAE as well as the European Society of Endodontology supports the use of Ca(OH)_2 as an intracanal medicament during endodontic regeneration [202]. However, this study as well as others should be taken into account when considering disinfection during REPs; the lack of residual antibacterial efficacy of Ca(OH)_2 cannot be overstated. The desired property of a canal disinfectant is to render the canal aseptic, and provide an environment favorable for growth. This includes sustaining an aseptic environment until root development can happen on its own, a condition that must be appreciated.

Some limitations of this study are the sample isolates. This study was conducted by a single practitioner, utilizing single isolates of both a mature and an immature tooth with pulpal necrosis. Although this study has agreement with other clinical studies similar to it, more clinical isolates would be beneficial to the strength of this study. More studies are encouraged to confirm the findings stated above. Although strict protocol was followed for handling and culturing the clinically isolated bacteria, it is possible to speculate that some bacteria may have been lost during the transfer from patient to laboratory. Additionally, as stated, one advantage of the methylcellulose being added to the DAP is its ability to adhere to the dentin and provide a homogenous concentration of antibiotics. This can also be a limitation of the paste because its handling characteristics

are not as easy as $\text{Ca}(\text{OH})_2$. The development of an intracanal delivery tip has not yet been produced.

SUMMARY AND CONCLUSIONS

My hypothesis, which stated that all tested concentrations of DAP will not prevent the formation of multi-species biofilms regardless of the biofilm source, was partially accepted. In conclusion, this study suggested that only 5 mg/mL of DAP was able to provide significant residual antibacterial effect against bacterial biofilms from an immature tooth with pulpal necrosis. Ca(OH)_2 failed to provide any residual antibacterial effect once it was removed from the dentin samples.

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ABSTRACT

THE ABILITY OF NEW INTRACANAL MEDICAMENTS TO PREVENT THE
FORMATION OF MULTI-SPECIES BIOFILM
ON RADICULAR DENTIN

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We explored the residual antibacterial properties of dentin pretreated with low concentrations of double antibiotic paste (DAP) against biofilm bacteria obtained from different clinical sources. Dentin blocks were sterilized and randomized into four treatment groups and two control groups (n = 20). Blocks from treatment groups were pretreated with DAP (1 mg/ml or 5 mg/ml) loaded into methylcellulose, calcium

hydroxide ($\text{Ca}(\text{OH})_2$), or methylcellulose paste. After one week, the treatment pastes were removed and all blocks were immersed in PBS. The dentin blocks from treatment groups and one of the control groups were then inoculated with bacterial isolates obtained from immature or mature teeth with pulpal necrosis ($n = 10$). The remaining control group received no bacteria and was used as a sterile control. Blocks were then incubated anaerobically for 3 weeks. Biofilm disruption assays were conducted for all samples. Two-way ANOVA and pair-wise comparisons were used for statistical analyses. The residual antibacterial effect of dentin pretreated with 5 mg/ml of DAP was significantly greater than all other groups regardless of the source of biofilm. Dentin pretreated with 1 mg/ml of DAP demonstrated significantly greater residual antibacterial effects in comparison with dentin pretreated with placebo paste and $\text{Ca}(\text{OH})_2$ only in bacterial isolates obtained from mature teeth with pulpal necrosis. Dentin pretreated with $\text{Ca}(\text{OH})_2$ did not demonstrate any residual antibacterial effects. Dentin pretreated with 1 mg/ml or 5 mg/ml of DAP demonstrated significantly better residual antibacterial effects against biofilm bacteria obtained from mature teeth with pulpal necrosis in comparison with bacterial isolates obtained from immature teeth with pulpal necrosis.